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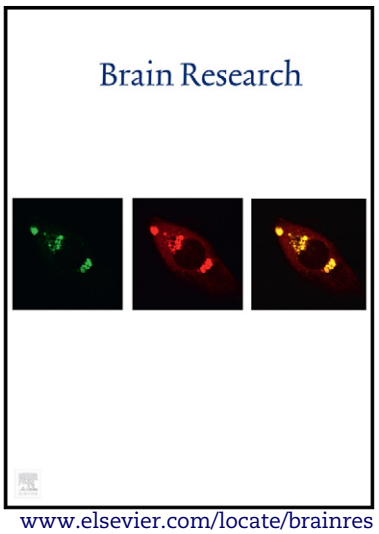

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Developmental exposure to ethanol increases the neuronal vulnerability to oxygen-glucose deprivation in cerebellar granule cell cultures

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Abstract

Prenatal alcohol exposure is associated with microencephaly, cognitive and behavioural deficits, and growth retardation. Some of the mechanisms of ethanol-induced injury, such as high level oxidative stress and overexpression of pro-apoptotic genes, can increase the sensitivity of fetal neurons towards hypoxic/ischemic stress associated with normal labour. Thus, alcohol-induced sequelae may be the cumulative result of direct ethanol toxicity and increased neuronal vulnerability towards metabolic stressors, including hypoxia. We examined the effects of ethanol exposure on the fetal cerebellar granular neurons' susceptibility to hypoxic/hypoglycemic damage. A chronic ethanol exposure covered the entire prenatal period and 5 days postpartum through breastfeeding, a time interval partially extending into the third-trimester equivalent in humans. After a binge-like alcohol exposure at postnatal day 5, glutamatergic cerebellar granule neurons were cultured and grown for 7 days *in vitro*, then exposed to a 3-hour oxygen-glucose deprivation to mimic a hypoxic/ischemic condition. Cellular viability was monitored by dynamic recording of propidium iodide fluorescence over 20 hours reoxygenation. We explored differentially expressed genes on microarray data from a mouse embryonic ethanol-exposure model and validated these by real-time PCR on the present model. In the ethanol-treated cerebellar granule neurons we find an increased expression of genes related to apoptosis (*Mapk8* and *Bax*), but also of genes previously described as neuroprotective (*Dhcr24* and *Bdnf*), which might suggest an actively maintained viability. Our data suggest that neurons exposed to ethanol during development are more vulnerable to *in vitro* hypoxia/hypoglycemia and have higher intrinsic death susceptibility than unexposed neurons.

Highlights:

- Fetal ethanol exposure increases neurons' vulnerability towards metabolic stressors
- Maternal alcoholism decreases offsprings' neuronal tolerance to hypoxia/ischemia
- Ethanol alters expression of genes associated with apoptosis and neuroprotection

Keywords: *prenatal ethanol exposure; oxygen-glucose deprivation; cerebellum; neuronal vulnerability*; *differential gene expression.*

1 Introduction

Prenatal alcohol exposure secondary to maternal ethanol consumption causes deleterious effects on fetal brain development (Falk, 2008). Even brief exposure to ethanol can result in growth retardation, craniofacial (Ismail et al., 2010) and systemic congenital abnormalities (Stratton, 1996). The damaging effects of ethanol on the developing nervous system have been thoroughly described since fetal alcohol syndrome was first investigated (Jones and Smith, 1975). The extent of neuronal impairment can lead accordingly to intellectual deficits, seizures, and even paraplegia (Streissguth and O'Malley, 2000). Fetal ethanol exposure determines an attenuation of hypoxic vasodilation that limits fetal oxygen delivery to the brain during hypoxic episodes (Mayock et al., 2007) and leads to altered glucose transport and metabolism (Fattoretti et al., 2003), promoting cellular damage during physiological peripartum hypoxia (Bakker and van Geijn, 2008). Moreover, chronic binge alcohol consumption has been shown to alter maternal uterine vascular function, leading to lowered blood supply to the placenta and consequently to the fetus (Subramanian et al., 2014).

The cerebellum is highly sensitive to both, ethanol (Goodlett and Eilers, 1997) and hypoxia/ischemia (Cervos-Navarro et al., 1991; Goodlett and Eilers, 1997). Ethanol exposure during development causes an increased apoptotic cell death rate of both, cerebellar Purkinje and granule cells (Bhave and Hoffman, 1997), responsible for the severe motor impairments commonly associated with cerebellar dysfunction

Abbreviations

BAL, blood alcohol level; CGCs, cerebellar granule cells; C_T, cycle threshold; Ctr, control; EM+/-G, experimental medium with/without glucose; EtOH, ethanol; GO, gene ontology; OGD, oxygen-glucose deprivation; PI, propidium iodide; qPCR, quantitative polymerase chain reaction.

(Goodlett and Eilers, 1997). Neonatal cerebellum in rats shows a clear temporal window of vulnerability during the first 10 days after birth, a period corresponding to Purkinje cell dendritic outgrowth and synaptogenesis and to the third pregnancy trimester in humans (Dobbing and Sands, 1979; Goodlett et al., 1990). Within this period, cerebellar granule cells (CGCs) display a higher susceptibility to ethanol on postnatal days 4-6 (Goodlett et al., 1998), when proliferation of CGCs precursors towards postmitotic cells still occurs (Luo, 2012) and even short ethanol exposures can lead to significant loss of cerebellar neurons by intricate mechanisms such as inhibition of neurotrophic action, decrease in the pro-survival CREB binding protein expression (Guo et al., 2011), increased oxidative stress (Kotch et al., 1995), and activation of pro-apoptotic pathways (Light et al., 2002).

We investigated whether and how ethanol exposure influences the neuronal response to hypoxia/ischemia. Thus, CGCs cultures obtained from pups exposed to ethanol during fetal life and until postnatal day 5 were used in an *in vitro* oxygen-glucose deprivation (OGD) model. The model allows the evaluation of neurons' *in vitro* maturation after an *in vivo* ethanol exposure, which intercepts the maximum cerebellar vulnerability to ethanol, before the completion of neurite formation. Acute and delayed neuronal vulnerability secondary to OGD was assessed by a 20 hours-dynamic measurement of cellular death using propidium iodide (PI) fluorometry. Our data suggest that neurons exposed to ethanol during development are more vulnerable to *in vitro* hypoxia/hypoglycemia. To check whether our results are confirmed on a molecular level and to support a possible *in vivo* extrapolation of the results, we further analyzed gene expression profiles from publicly available microarray data in a mouse model of embryonic ethanol exposure. This revealed several biological pathways that showed differential regulation after ethanol exposure, including

100 embryonic organ and neuronal morphogenesis. Additionally, we validated genes by
101 real-time PCR on the *in vitro* CGC model and identified some candidate genes which
102 could be responsible for the low adaptive response to hypoxia/hypoglycemia of
103 cerebellar granule cells derived from pups exposed to ethanol during the brain growth
104 spurt period.

105 **2 Results**

106 *2.1 Morphological assessment*

107 Morphology and cell maturation of cultured CGCs were assessed in phase-contrast
108 microscopy. There were no obvious morphological differences between cultures
109 originating from ethanol-treated and non-treated animals (Fig. 1A). The
110 morphological assessment at the end of the reoxygenation protocol revealed the
111 nature and the extent of damage induced by OGD and/or ethanol pretreatment (Fig.
112 1B). Phase-contrast microscopy showed an increased level of cell injury in ethanol-
113 exposed neurons, ranging from cytoplasmic swelling, loss of phase-bright contours,
114 and intercellular connections in ethanol control group cultures, to loss of membrane
115 integrity and the presence of multiple cell ghosts in the ethanol-treated cultures
116 exposed to OGD (Fig. 1B). Cell death was confirmed on the same microscopic fields
117 by PI fluorescence (Fig. 1B).

118 *2.2 The response of ethanol-treated neurons to oxygen-glucose deprivation*

119 We first assessed the effects of ethanol pre-exposure on the neuronal response to an
120 OGD protocol. Neuronal viability at the end of a 3-h OGD exposure before the
121 initiation of reoxygenation was measured by PI fluorescence. Figure 2A indicates that
122 ethanol-treated neurons showed a significantly higher susceptibility to OGD exposure
123 (7.0 ± 2.0% cell death, n = 5) comparing to non-treated neurons exposed to OGD

(0.65 ± 0.10% cell death, n = 4), p-value = 0.03. In the groups maintained in control normoxic/normoglycemic conditions, there was also a small but significant difference in survival between the non-treated control neurons and ethanol control neurons (0.26 ± 0.05% cell death, n = 4 and 0.62 ± 0.09% cell death, n = 6, respectively, p-value = 0.01).

Reoxygenation at the end of the OGD period resulted in additional neuronal death, with an increased death in the non-treated population at 20 h of reoxygenation (Fig. 2B). Figure 2B also shows that the increased neuronal vulnerability, as an effect of ethanol pre-exposure, is maintained over the 20-h period of reoxygenation following OGD (33.4 ± 2.3% cell death, n = 5 for the ethanol-treated neurons, vs. 22.4 ± 1.1% cell death, n = 4 for the non-treated neurons, p-value = 0.004).

2.3 Dynamic recording of neuronal death during reoxygenation

Neuronal delayed death was dynamically recorded for 20 h by PI signal to gain further insight into how ethanol pre-exposure affects neuronal susceptibility to different levels of metabolic deficits. Figure 3A provides typical examples of such recordings for each of the conditions used in this study. Maintenance of the primary neuronal cultures for 20 h in the low-nutrient PI-containing experimental medium could result in metabolic stress, which induced a low, but constant rate of neuronal cell death in control cultures. For the first few hours of PI-signal recording, there was no significant difference between the ethanol-treated and the non-treated neurons. The sensitising effect of ethanol pretreatment became manifest only after 10-11 h in control cultures. In case of the more demanding metabolic stress induced by OGD, the higher death vulnerability of the ethanol-treated neurons was clearly manifest from the beginning of reoxygenation period (Fig. 3A). These results suggest that neurons from ethanol-exposed pups have an additional susceptibility to hypoxic-ischemic

challenge that is separate from the toxic effect of ethanol alone. Figures 3B and 3C illustrate these differences across the experimental set using an hourly rate of neuronal cell death as the measured parameter. Figure 3B shows the sensitising effect of ethanol pretreatment becoming manifest only in the latter part of the experimental protocol in control cultures (p-value < 0.001, n = 6 ethanol-treated control group and n = 4 non-treated control group).

After OGD exposure, ethanol-treated cultures showed a significantly different vulnerability in comparison to control cultures (p-value < 0.001, n = 4 for non-treated group and n = 5 for ethanol-treated group). OGD induced extensive damage in the ethanol group during early reoxigenation, as shown by hourly cell death rate 2.9 ± 0.4%/hour compared to ethanol non-treated cultures 0.73 ± 0.17%/hour (Fig. 3C). Given the significant death of the ethanol-treated neurons during the first part of the dynamic recording, hourly cell death rate in late reoxigenation was higher in the control group (Fig. 3C), but overall OGD-induced cell death was still higher in the ethanol group at the end of reoxagenation (66.5 ± 2.9% vs. 41.5 ± 2.1%) (Fig. 3A).

2.4 Microarray gene expression in control and ethanol-exposed mouse embryos

In the attempt to understand the molecular mechanism which renders ethanol-treated neurons more vulnerable to metabolic stresses, we analized gene expression data available on a public-domain microarray dataset (GSE9545 from Gene Expression Omnibus Database), which provides the nearest model example to our experimental model. In this experiment whole embryo mouse cultures were performed on four control embryos and four ethanol-treated ones, the latter characterized by a phenotype of open neural tubes (Wang et al., 2008). Transcriptomic studies suggest that teratogenic effects observed in whole embryo cultures are relevant to previously

identified mechanisms of toxicity *in vivo* (Genschow et al., 2002; Luijten et al., 2010; Zhou et al., 2011).

Given our *in vitro* observations suggesting a metabolic deficiency induced by ethanol exposure of the offspring, we assessed the effect of ethanol exposure on whole embryo changes in gene expression in various sets of relevant genes. Thus, informed by our *in vitro* data we used an additional model as a probe for the potential changes. Validation of the observed vulnerability on a molecular level in the whole embryo cultures model would provide an important link to the *in vivo* developmental toxicity (Robinson et al., 2012a).

A widely used systems biology technique to highlight biological processes is gene category over-representation analysis (Park et al., 2014). To perform this analysis genes are grouped into categories by a common biological property and then tested to find categories that are over-represented. To this end gene ontology (GO) pathways (Ashburner et al., 2000) that are affected by exposure to ethanol were investigated. After removing genes which were not reliably detected, we ranked and clustered 16,117 genes according to the expression fold change between the ethanol-exposed and control embryos. Genes clustered in 161 GO categories after correcting for multiple testing by the stringent method of family-wise error rate (FWER < 0.01) (Krzywinski and Altman, 2014). Categories that could be relevant to the higher susceptibility towards metabolic stressors secondary to ethanol exposure of the embryo were further analyzed (Table 1).

To identify genes that might be responsible for the observed neuronal susceptibility towards metabolic stressors of ethanol-exposed embryos we first screened the GO category "oxidation-reduction process" (GO:0055114, p-value = 8.80e-08,

FWER < 1.0e-04). This category had an enrichment in genes that were low ranked according to the fold change between the ethanol-exposed group and control, which is translated in a higher number of down-regulated genes than expected by chance. *Dhcr24* (gene coding 24-dehydrocholesterol reductase, p-value = 0.006, fold change = 1.13) and *Cp* (gene coding ceruloplasmin, p-value < 0.001, fold change = 0.71) were the most significantly up and down-regulated, respectively (Table 2).

The category "mitochondrial respiratory chain" (GO:0005746, p-value = 6.27e-06, FWER = 0.003) was enriched in genes that were low ranked, but among the 45 genes in the node none fulfilled the fold change criterion of up- / down-regulation by at least 10%.

Genes that might be responsible for the developmental effects of ethanol on embryos were revealed by focusing on two GO biological processes: "*in utero* embryonic development" (p-value = 2.04e-06, FWER = 0.003) and "nervous system development (p-value = 5.5e-08, FWER = 0.0001)". This resulted in 4 significantly up-regulated genes involved in embryonic development (GO:0001701) (Table 2), of which *Nrk* (gene coding Nik-related protein kinase, p–value = 0.02, fold change = 1.18) was the most significant. Additionally, 7 genes clustering in this category (Table 2) were significantly down-regulated, with *Slit2* (gene coding Slit homolog 2 protein, p-value = 0.002, fold change = 0.89) most significant. Significantly differentially expressed genes, which clustered in the nervous system development category (GO:0007399) are presented in Table 2, with 19 up-regulated and 21 down-regulated representatives. Of these the most significant up- / down-regulated ones were respectively: *Pitx1* (gene coding paired-like homeodomain

transcription factor 1, p-value = 0.003, fold change = 1.13) and *Slit2*, which clustered in both the previous and this category.

2.5 Gene expression quantification on cerebellar granule neurons cultures from ethanol-exposed and control rat offsprings

To verify which genes could be responsible for the higher vulnerability of ethanol-treated neurons to metabolic stresses, we quantified gene expression levels for candidates obtained from the microarray analysis. The overlap of differentially expressed genes on the CGCs model and whole embryo cultures lends further support to the validity of the identified candidate genes.

We validated five genes as possible candidates: *Dhcr24*, *Bdnf*, *Mapk8*, *Bax*, and *Slc2a4* (Fig. 4). It is common that given the different techniques, qPCR and microarray measurements show different fold changes of the genes, even when the same samples are measured (Lussier et al., 2015). In the case of *Dhcr24* the fold change between the ethanol group and the control is 23 ($\Delta\Delta C_T = 4.55$), while the microarray data showed an increase of only 1.13 fold. *Bdnf* shows a marked up-regulation of 32 fold ($\Delta\Delta C_T = 5.04$), while in the microarray data the gene is found to be down-regulated in the ethanol group (fold change = 0.81, Table 2).

Although the last three genes did not show differential expression in the whole mouse embryo culture model, the rationelle for their measurement was the involvement in the GO categories which showed disruption and may be relevant for the ethanol-induced increased susceptibility towards metabolic stressors (Table 1). While MAPK8 interacting proteins 1 and 2 showed an up regulation after ethanol exposure (Table 2, GO:0032872), *Mapk8* showed no significant regulation (fold change = 1.04). Our measurements revealed a 7.1 fold up-regulation of *Mapk8* in the

ethanol group ($\Delta\Delta C_T = 2.82$). *Bax* and *Slc2a4* were also up-regulated in the ethanol group samples to 7.95 fold and 41 fold, respectively ($\Delta\Delta C_{T\ Bax} = 2.99$ and $\Delta\Delta C_{T\ Slc2a4} = 5.39$).

Genes involved in the oxidative stress response (*Cp*, *Snca*, *Gss*) failed to reach significance in the qPCR measurements.

3 Discussion

Our study indicates that ethanol exposure during nervous system development results in a significantly higher neuronal vulnerability to metabolically demanding conditions, either under chronic conditions, such as maintenance in a low nutrient medium, or in more acute conditions, such as OGD, which is the *in vitro* equivalent of hypoxia/ischemia.

In their review, Bosco and Diaz hypothesised that ethanol-induced fetal growth retardation occurs most likely as a result of hypoxia and increased oxidative/nitrative stress, which interfere with cellular processes that require oxygen in order to function adequately, such as placental transport (Bosco and Diaz, 2012). Although tested only on one type of cells, the *in vitro* CGCs results support this hypothesis since ethanol exposure during brain growth spurt aggravates the cellular damage induced by a hypoxic/ischemic injury (Figs. 2 and 3).

To validate the ethanol-induced vulnerability in an additional model, but also to understand the mechanisms that underlie the ethanol-sensitizing effect to both chronic and acute metabolic challenges, we performed gene expression data analysis on a publicly available microarray dataset from whole embryo cell cultures. Whole embryo cell cultures show gene expression patterns very similar, over time, to *in utero* embryos; and, in the case of rat, cultures are matching to a great extent the gene expression profile in human embryos undergoing neurulation and early embryogenesis (Robinson et al., 2012b). Therefore, given the high *in vivo* translation potential of whole embryo cell cultures (Genschow et al., 2002), the results can benefit of better support for a possible *in vivo* extrapolation. However, whole embryos display tissue heterogeneity, which may mask some changes in specific tissues or cells (Zhou et al., 2011). Hence, we validated some of the candidate genes on the *in*

276 *vitro* model of CGCs cultures originating from offspring exposed to ethanol during
277 cerebellar development.
278 We assessed expression patterns of genes involved in pathways correlated with a
279 higher metabolic vulnerability. Mitochondrial dysfunction plays a key role in hypoxic
280 neuronal injury and could therefore be a determining factor in the CGCs' damage
281 caused by ethanol preexposure. Upon inquiry of the "mitochondrial respiratory chain"
282 GO category, there was a significant enrichement with down-regulated genes, but
283 given the strict cutoffs set for candidate genes, none of the genes in this cluster
284 reached the threshold for significance. Since a strict cut-off to limit false-positive
285 results leads to an increase of the number of false negative results (Kowalchuk and
286 Keselman, 2001), and also because oxidative stress has been recently described as an
287 important factor in the pathophysiology of alcohol-induced impairment (Joya et al.,
288 2014), we further investigated other related pathways.
289 A close inspection of the GO category "oxidation-reduction process" revealed an
290 ethanol-induced up-regulation of *Dhcr24* shown to exert a neuroprotective effect
291 against reactive oxygen species resulted from endoplasmic reticulum stress (Lu et al.,
292 2014). The down-regulation of *Cp*, an important antioxidant molecule, is in
293 accordance to previous studies on hippocampi after chronic ethanol exposure (Saito et
294 al., 2002). Another down-regulated gene of the pathway is *Snca*, a synaptic molecule
295 involved in neurodegenerative disorders and an oxidative stress protector of neuronal
296 cells (Hashimoto et al., 2002). While *Dhcr24* was verified by qPCR on the CGCs
297 model, *Cp* and *Snca* failed to reach significance. Still, we cannot exclude that on the
298 whole embryo level oxidative stress plays an important role in the ethanol-induced
299 pathology. However, the proapoptotic mitochondrial-membrane associated *Bax*
300 showed an elevated expression in the ethanol group on CGCs. It has recently been

shown that ethanol exposure of rats on postanatal day 4 leads to an increased activation of BAX (Heaton et al., 2015), which together with high expression levels of the gene, could lead to apoptosis. Nonetheless, under basal conditions we could not detect morphologically higher apoptotic levels in the ethanol group (Figures 1 and 3A). Overexpression of *Dhcr24* was shown to inhibit apoptotic cell signaling, hence it seems reasonable to assume that the observed upregulation of *Dhcr24* is a counterbalance that prevents the commitment on the activated apoptotic pathway in CGCs.

Additionally, it is notable that *Bdnf*, a gene that appears down-regulated following ethanol exposure of embryos, can have protective effects on cerebellar granule neurons under low glucose conditions (Vakili Zahir et al., 2012). *Bdnf* was shown to prevent JNK and p38 activation in stress conditions and thus increase cell viability. The CGCs *in vitro* expression data confirm the overexpression of *Mapk8* (*Jnk1*), which is known to promote apoptosis (Dhanasekaran and Reddy, 2008). Conversely, *Bdnf* is up-regulated after ethanol exposure in the CGCs model, unlike on whole embryo cultures. It seems likely that up-regulation of both genes maintains a balance in the basal viability and we hence observe a higher vulnerability only under challenging conditions.

We show that *Slc2a4*, the transcript coding for the insulin-sensitive glucose transporter GLUT4, is up-regulated after ethanol exposure in CGCs. GLUT4 was described to be present in significant amounts in CGCs and was shown to be up-regulated in the CGCs of a diabetic, hyperinsulinemic mouse model (Vannucci et al., 1998). CGCs showed an opposite expression profile in the diabetic mice when compared to periferic tissues in which *GLUT4* transcript was decreased. Additionally, prenatal alcohol exposure induces impaired glucose tolerance (Chen et al., 1996).

Thus, *Slc2a4* might be a key candidate to explain the impaired cerebellar glucose metabolism and high susceptibility to *in vitro* hypoxia and hypoglicemia in cultured CGCs from prenatally ethanol-exposed rats.

Adm, a shared down-regulated gene between "embryonic and nervous system development" GO categories, was shown to lead to lower resistance to hypobaric hypoxia in mice that were *Adm* conditional knockouts in the central nervous system (Fernandez et al., 2008). Moreover evidence points towards an involvement of this gene in placentation and regulation of fetal perfusion (Wilson et al., 2004), which together with the known ethanol cytotoxic effects on trophoblast cells (Clave et al., 2014) may support an altered placental function in ethanol drinking dams. Although not significantly disrupted in CGCs, the expression of the *Adm* gene seems to be more relevant on an entire organism level, such that mild impairment of the placental function could result in decreased neuronal viability.

Taken together ethanol might confer a higher risk towards hypoxic/ischemic events, which can lead to growth retardation as suggested by Bosco and Diaz (Bosco and Diaz, 2012) given the confirmed predisposition towards hypoxia and glucose deprivation in the neurons. Furthermore, in CGCs, survival was reduced, with a certain lag time, even under less severe metabolic conditions, such as the *in vitro* maintenance of neurons in an artificial environment, with a relative reduction in the supply of nutrients (Fig. 3). We show that in our experimental model genes involved in apoptosis have a higher expression level after ethanol treatment. However, compensatory mechanisms involving neuroprotective genes, which we show to be up-regulated, are probably responsible for the apparent similar viability between the ethanol-exposed and control neurons.

In sum, the present results suggest an ethanol-induced reduction of the cellular adaptation to stress, which was verified in both, the *in vitro* CGCs cultures and on a molecular level in whole embryo cultures. The gene profiles and the observed pattern of ethanol-treated neurons impairment should both together contribute to the generation of *in vivo* studies concerning secondary prevention of brain damage, such as avoiding additional mild hypoxic events or metabolic stressful circumstances in the newborn.

4 Experimental Procedure

4.1 Animals

Sixteen pregnant Wistar rats and thirty-one of their litters from the breeding colony at Carol Davila University of Medicine and Pharmacy were used in this study. All animal procedures were carried out with the approval of the local ethics committee for animal research of Carol Davila University of Medicine and Pharmacy (Bucharest, Romania), and in accordance with the European Communities Council Directive 86/609/EEC on the protection of animals used for scientific purposes. All efforts were made to minimize the number of animals used and their suffering.

4.2 Ethanol treatment

Pregnant rats were randomly assigned on the day of confirming pregnancy, to either an ethanol group or a non-treated group and housed separately thereafter. Both groups had free access to food and liquids, and the ethanol group dams were given ethanol 20% v/v in the drinking water to induce ethanol exposure damage to the litter (Snyder et al., 1992). To validate the level of ethanol exposure, blood alcohol levels (BAL) were randomly measured during the entire setup time frame using an enzymatic, UV method (Dialab, Austria). This type of exposure resulted in maternal BAL of 112.7 ± 19.3 mg/dL ($n = 6$) (data represent mean value ± SEM). Nutrition and drinking patterns were daily monitored for both groups, and no nutritional deficits were observed. Mean block-food ingestion did not significantly differ between ethanol treated and non-treated group, with an average calorie intake of 269.1 ± 6.8 kcal/kg body weight/day (data represent mean value ± SEM). Liquids consumption in the non-treated group was 129.2 ± 5.3 ml/kg body weight/day. The fluid ingestion in the ethanol treated group was 139.6 ± 9.5 ml/kg body weight/day, of

382 which an average of 22.0 ± 1.6 g ethanol/kg body weight/day. The body weight did
383 not differ significantly between the two groups at different pregnancy stages.
384 To mimic ethanol exposure during the third trimester of pregnancy in humans, full-
385 term litters, known to correspond developmentally to the end of the second trimester
386 in humans, were further exposed to ethanol through lactation until postnatal day 5, as
387 the dams continued to receive ethanol 20% v/v in the drinking water (Olney et al.,
388 2000). BAL was monitored during this period, by random serum measurements, and
389 reached values of 109.7 ± 43.9 mg/dl in pups (n = 4). As ethanol exposure through
390 breastfeeding might not be sufficient to induce a similar to human third-trimester
391 exposure causing cerebellar disfunction (Cebolla et al., 2009), postnatal day 5 pups
392 were exposed to a "binge-like" pattern of ethanol consumption, common in women
393 who drink during pregnancy (Maier et al., 1997). This ethanol acute exposure was
394 mimicked by a 3-hour vapor inhalation (Heaton et al., 2000b), resulting in a BAL of
395 461.5 ± 165.8 mg/dl (n = 4) in pups, a concentration comparable to the high levels
396 seen in previous human and animal studies (Tran et al., 2005). The chronic and acute
397 combinatorial model of ethanol exposure corresponds to the behavioral pattern of
398 steady drinkers (Epstein et al., 1995). Cerebella did not significantly differ in either
399 morphology or weight compared to unexposed pups.

400 *4.3 Cerebellar granule neurone cultures and oxygen-glucose deprivation exposure*

401 The exposure model was further transferred into an *in vitro* setup allowing a better
402 assessment of the deleterious effects which ethanol exerts on cerebellar granule
403 neurons. CGCs cultures are widely used for the *in vitro* study of neuronal ischemia
404 (Kalda et al., 1998) or ethanol neurotoxicity (Heaton et al., 2000a; Luo, 2012) since
405 they provide a homogenous population of glutamatergic neurons in which
406 experimental conditions can be precisely controlled (Contestabile, 2002). Primary

cultures of cerebellar granule neurons were obtained as previously described (Toescu, 1999) immediately after the acute ethanol exposure or a sham air exposure, for ethanol and non-treated pups, respectively. Briefly, the dissociated cell suspensions from cerebella of 5-day-old pups were plated at a density of 200,000 cells/well into 96-well plates (Nunc) coated with poly-D-lysine (Sigma-Aldrich) in complete BME culture medium (Sigma-Aldrich) containing 2 mM L-glutamine (HyClone), 32 mM glucose, 10% heat-inactivated horse serum (Sigma-Aldrich), antibiotic-antimycotic (GIBCO/Invitrogen) and 25 mM KCl, to promote CGCs viability in culture (Gallo et al., 1987). Cytosine-arabinoside (Sigma-Aldrich, 10 μM) was added after 24 h to stop the glial cells proliferation. There were no apparent differences between the cultures generated from ethanol-treated group and the cultures from the non-treated grup, in regard to morphology or number of viable neurons. Cultures were grown in a humidified incubator at 37°C and 5% CO_2 for 7 days until they reached a maturation level that allowed evaluating different experimental conditions. The percentage of glial cells was no more than 1% as assessed by glial fibrillary acidic protein and β-tubulin immunostaining. The cultures were grown in ethanol-free solutions to promote similar adaptation to *in vitro* state for both groups. Control condition and OGD which mimics ischemia *in vitro* (Goldberg et al., 1986), were carried out as previously described (Ceanga et al., 2010), by shifting cultures in a serum-free experimental medium (EM) with or without glucose (EM+G, EM-G, containing in mM: 120 NaCl, 25 KCl, 0.62 $MgSO_4$, 1.8 $CaCl_2$, 10 HEPES, ± 11.1 glucose, pH 7.4). Briefly, OGD was initiated by washing cultures with EM-G to remove glucose before adding deoxygenated EM-G. Then, cultures were immediately placed into a humidified hypoxia chamber (Billups-Rothenberg Inc., Del Mar, CA) which was flushed with 100% N_2 for 10 min, sealed, and placed into an incubator at 37°C for 3 h,

an OGD exposure time that we previously found to induce neuronal damage (Zagrean A.M., 2007-Personal Communication). Reoxygenation was induced by replacing EM-G with normoxic EM+G. Control cultures went through identical steps except they were kept in normoxic EM+G in a 5% CO_2 incubator at 37°C (control condition).

4.4 Experimental groups

Four experimental groups were defined to evaluate the neuronal vulnerability to a metabolic stress, considering both the *in vivo* ethanol treatment and the *in vitro* exposure of CGCs cultures to OGD or control conditions: two ethanol-treated groups, an ethanol OGD group and an ethanol control group, and two non-treated groups, a non-treated OGD group and a non-treated control group.

4.5 Assessment of cellular viability

4.5.1. Microscopic examination

Morphology and cell maturation were assessed in phase-contrast microscopy with a Zeiss Axiovert25 inverted microscope at 400× magnification during the cultures' growth period and no morphological difference between ethanol-exposed neurons and neurons from non-ethanol treated pups were observed. Cellular morphology was also assessed immediately after OGD or control conditions. The wells containing disrupted cultures were discarded. The cellular damage induced by ethanol pretreatment and OGD was further morphologicaly assessed in phase-contrast microscopy at the end of reoxygenation period.

4.5.2. PI fluorometry

The rate of delayed cell death was monitored for the next 20 hours after control and OGD conditions by use of a vital dye, PI (Sattler et al., 1997), which becomes fluorescent upon binding the DNA of membrane-compromised cells (Vornov et al.,

1995). Cells were washed once with 200 µl EM+G immediately after OGD or control conditions, then 100 µl PI solution (50 µg/ml in EM+G) was added in each well. The initial level of PI fluorescence in a sequence of five readings at 60 seconds interval was read in a multimode detector (DTX880, Beckman Coulter) set in fluorescent mode with an excitation wavelength of 535 nm and an emission wavelength of 625 nm. Dynamic PI measurements were initiated at every 15 minutes for 20 hours of reoxygenation at 37°C. At the end of reoxygenation time, the PI solution was exchanged with 25 µl ethanol (100%)/well to induce maximal death. After ethanol evaporation the PI solution was added back and the maximal signal (corresponding to the total number of cells) was measured in a sequence of 5 readings at 60 second intervals.

4.6 Microarray gene expression analysis

Microarray expression data was obtained from the public database Gene Expression Omnibus under the accession number GSE9545. We analyzed the dataset that measured the global gene expression profiles in whole mouse embryo cultures, comparing control with alcohol treated embryos that had a clear phenotype which consisted in open neural tubes. A full description of the experimental ethanol model is available in the study of Wang et. al (Wang et al., 2008). Microarray expression was calculated using the `affy` (Gautier et al., 2004) and `gcrma` (Wu et al., 2004) of the `Bioconductor` (Gentleman et al., 2004) extensions to the `R` statistical programming environment (http://www.R-project.org). Genes that were not reliably detected in at least one of the two conditions were removed from further analysis. To identify metabolic pathways influenced by ethanol exposure genes were clustered in gene ontology categories (Ashburner et al., 2000) using `FUNC` package (Prufer et al., 2007) with a Wilcoxon ranking test according to the fold change of gene expression.

Family-wise error rate (< 0.01) was used to correct for multiple testing hypothesis (Krzywinski and Altman, 2014). On the gene expression level a two sample *t*-test was used for statistical analysis. The MicroArray Quality Control (MAQC) project has shown that results of typical statistical differential expression tests with p-value as significance threshold need to be filtered and sorted by effect strength (fold-change) to attain robust comparisons across platforms and sites (Consortium et al., 2006). Hence, genes were defined as candidates if they clustered in a significant GO category, had a p-value < 0.05 and a fold change between the ethanol-treated and control group either lower than 0.9, or higher than 1.1. Significance level of genes in the analyzed GO categories was established according to p-value and fold change between the ethanol group and control.

4.7 Cerebellar granule neurons cDNA preparation and quantification by qPCR

CGCs originating from rat pups exposed to ethanol, or under control conditions were obtained as described above. Offsprings originated from two dams for each group, cerebella were collected, three and four cell cultures were made for the control and ethanol-group, respectively. Total RNA was isolated using 500 µl TRI REAGENT™ (Sigma Aldrich) according to the manufacturer's instructions. 550 ng of RNA were reverse transcribed (Superscript, Invitrogen™) with oligo(dT) primer in a total reaction volume of 20 µl. 1 µl of cDNA was further subjected to real-time PCR using Platinum-SYBR Green qPCR Supermix (Invitrogen), forward and reverse primers (0.9 µM), and ROX (100 nM, 5-carboxy-X-rhodamine, passive reference dye). Primers were designed with the Primer3 software for the following genes: *Cp* (forward primer: 5'–CATGTGGATGCTCCAAAAGA–3', reverse primer: 5'–GGTTCCGAGCAGAAGGTTTT–3'), *Dhcr24* (forward primer: 5'–GCTCTCCCTCATCTTCGACA–3', reverse primer: 5'–

TGAGACAGTGAGCCATCCAG–3', *Adm* (forward primer: 5'–CTCGACACTTCCTCGCAGT–3', reverse primer: 5'–AGACGTGCTCTGCTTGTCCT–3'), *Bdnf* (forward primer: 5'–AGGAGCGTGACAACAATGTG-3', *Snca* (forward primer: 5'–AGAAAACCAAGCAGGGTGTG–3', reverse primer: 5'–CCCTCCACTGTCTTCTGAGC–3'), reverse primer: 5'–CGTGGACGTTTGCTTCTTTC–3'), *Mapk8* (forward primer: 5'–CGGAGATTCTACATTCACAGTCC–3', reverse primer: 5'–CGCTTAGCATGGGTCTGATT–3'), *Bax* (forward primer: 5–GCTGGACACTGGACTTCCTC–3', reverse primer: 5'–CTCAGCCCATCTTCTTCCAG–3'), *Slc2a4* (forward primer: 5'–GGCCGGGACACTATACCCTA–3', reverse primer: 5'–GCCAAGCACAGCTGAGAATA–3'), *Gss* (forward primer: 5'–AGGGGGTGTTGCTGAGGT–3', reverse primer: 5'–CTGGCTGACAGCATCTACCA–3'), and *B2m* (forward primer: 5'–ACATCCTGGCTCACACTGAA–3', reverse primer: 5'–CCGGATCTGGAGTTAAACTGG–3'). PCR was performed in an MX 3000P instrument (Stratagene, La Jolla, CA) using the following protocol: 5 min 50°C, 2 min 95°C, and 40 cycles of 15 s 95°C, 30 s 60°C. A product melting curve was recorded to confirm the presence of a single amplicon. The correct amplicon size was confirmed by agarose gel electrophoresis. Threshold (C_T) values were set within the exponential phase of the PCR. *B2m* shows constant level of expression in both ethanol-treated and control cell cultures (C_T values of 17.7 ± 0.5 and 17.9 ± 0.2, respectively), which supports its use as housekeeping gene (Butte et al., 2001). The housekeeping gene *B2m* (β2-microglobulin) was used for normalization

ΔC_T ($C_{T\,(gene)} - C_{T\,(B2m)}$) (Livak and Schmittgen, 2001). The relative expression levels are given as the difference between the ΔC_T corresponding to the non-treated and ethanol-treated groups, respectively, ($\Delta\Delta C_{T\,(gene)} = \Delta C_{T\,(gene\ Non\text{-}treated\ Group)} - \Delta C_{T\,(gene\ Ethanol\ Group)}$). Gene regulation ratios between the ethanol and control groups are given as $2^{\Delta\Delta CT}$ values (Livak and Schmittgen, 2001). Gene regulation was statistically evaluated by subjecting the ΔC_T values to a two-sided, unpaired Student's *t*-test.

4.8 Statistical analysis for CGCs cultures viability experiments

For each group, the experiments were performed at least 4 times, on independent CGCs cultures from litters of different dams, with multiple wells for each condition. Statistical analysis was performed considering the different litters as biological replicates, while corresponding number of culture wells were considered technical replicates. To account for both technical and biological variance we used a standard equation for error propagation, as described by Taylor:

$SD = \sqrt{SD_{biological\ replicates}{}^2 + SD_{technical\ replicates}{}^2}$ (Taylor, 1997). Numerical results are given as mean ± SEM.

To analyze whether cell death was influenced by time or assessment conditions, a multiple regression model was run. No interaction between general predictors was taken into account, as theory suggests there should be none. Into this we included conditions and time (covariate) as the two predictors. Prior to running the model we checked covariate and cell death for having approximately simetrical distributions (which was the case), and z-transformed covariate to a mean of zero and a standard deviation of one to achieve easier interpretable coefficients (Forstmeier and Schielzeth, 2011). We checked model diagnostics like distribution of residuals,

555 Cook's distance and dfbetas and none of these indicated outliers, or obvious
556 deviations from the assumptions of normality and homogeneity of residuals. The
557 model and most diagnostics were run using functions available in `R` (Team, 2011).
558 Generalized Variance Inflation Factors were derived using the function `vif` of the `R`
559 package `car` (Weisberg, 2011). Overall the two parameters used for analysis:
560 "assessment conditions" and "time", both influenced cell death at a signficant level
561 (comparison of full to the null model results in $F_{3,319} = 1163$, $p < 0.001$). The cell
562 death increased in time (estimate = 9.9613, SE = 0.2425, $t_{319} = 41.079$). Cell death
563 was also influenced by the assessment conditions as shown by comparing the full
564 model with a reduced one not comprising exposure conditions revealed and resulting
565 in an $F_{3,319} = 987.67$, and $p < 0.001$). This validated the data without running pair wise
566 comparisons and allowed for pair wise post-hoc comparisons to analyze the impact on
567 cell survival between different conditions. A value of $p < 0.05$ was considered
568 statistically significant.
569

Conflict of interest statement

The authors declare that they have no competing interests.

Authors contributions

DLD: Participated in the design of the research, experimental procedures, data analysis, microarray gene expression analysis, and manuscript writing.

AS: Participated in the design of the research, experimental procedures, and data analysis.

MC: Participated in data analysis.

LZ: Participated in manuscript writing.

TS: Participated in manuscript writing and qPCR experiments design.

ECT: Participated in data analysis and manuscript writing.

AMZ: Participated in the design of the research, experimental procedures, data analysis, and manuscript writing.

All authors have approved the final article manuscript.

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Figure legends:

Figure 1. Microscopy images of CGCs cultures originating from ethanol-treated or non-treated rat pups. A. Phase-contrast microscopy of CGCs cultures on *in vitro* day 7 before exposure to OGD and/or normoxic low nutrient medium used for control shows similar morphology in the etanol-treated and non-treated groups. **B.** Phase-contrast microscopy (upper pannel) and fluorescent (bottom pannel) images taken on the same field from non-treated and ethanol treated CGCs stained with propidium iodide, after 20 hours of reoxygenation following OGD or control conditions. Microphotographs were taken with a Zeiss Axiovert25 inverted microscope at 400x magnification. Scale bar represents 10 μm.

Figure 2. Cell death of ethanol-treated and non-treated cerebellar granule neurons exposed to OGD. A. At the beginning of reoxygenation cell death was found significantly increased in the ethanol-treated group exposed to OGD. Cell death was assessed by propidium iodide fluorescence. **B.** The effect of OGD on ethanol-treated and non-treated cultures after 20 hours of reoxygenation is expressed as % cell death difference between OGD and control cultures. At this time point, OGD-induced cell death remained significantly increased in the ethanol group, comparing with the non-treated group. Error bars represent SEM, $n \geq 4$ cell cultures from unrelated pups.

Figure 3. Cell death over 20 hours of reoxygenation in ethanol-exposed and non-treated cerebellar granule neurons subjected to OGD. A. OGD-induced higher cell death rates in ethanol-exposed neurons in comparison to non-treated ones. Under control conditions the difference between ethanol-exposed cells and non-treated ones became manifest after 10 hours of reoxygenation. Cell death was assessed every 15 minutes for 20 hours by propidium iodide fluorescence. **B.** In control cultures, hourly cell death rate calculated during early (2-7 h) and late (12-17 h) reoxygenation,

showed that the sensitising effect of ethanol pretreatment becomes manifest only during the late reoxygenation. **C.** OGD effect on hourly cell death rate, calculated as difference between OGD and control cultures, was significantly higher during early reoxygenation in ethanol-exposed neurons compared to non-treated ones. Due to the extensive damage during early reoxygenation in ethanol group, the hourly cell death rate during the late reoxygenation period became higher in non-treated cultures. Error bars represent SEM, $n \geq 4$ CGCs cell cultures from unrelated pups.

Figure 4. qPCR measurement of genes hypothesized to be influenced by prenatal alcohol exposure. Five genes are significantly up-regulated in the ethanol-exposed neurons compared to the non-treated ones. The measured genes are involved in the GO categories shown to be disrupted on a microarray dataset from whole embryo cell cultures after ethanol exposure (Tables 1 and 2). β2-microglobulin (*B2m*) expression levels are similar between the ethanol-exposed (17.7 ± 0.5) and non-treated CGCs cultures (17.9 ± 0.2),. C_T values for *B2m* were used for normalization ΔC_T ($C_{T\,(gene)}$ - $C_{T\,(B2m)}$). Error bars represent SEM, n = 3 and n = 4 for CGCs cultures of non-treated and ethanol-treated groups, respectively.

Table 1. Gene Ontology categories relevant for an increased susceptibility towards metabolic stressors observed in ethanol-exposed embryos. GO enrichment was tested using a Wilcoxon rank test from FUNC package, according to expression fold change between ethanol-exposed and control embryos. FWER = family wise error rate. First two nodes are enriched in low ranked genes and the others in high ranked ones.

Root node name	Node name	Node ID	No. of genes in node	raw p-value	FWER
biological process	oxidation-reduction process	GO:0055114	587	8.80E-08	< 1.0E-04
cellular component	mitochondrial respiratory chain	GO:0005746	45	6.27E-06	0.003
biological process	regulation of stress-activated MAPK cascade	GO:0032872	133	3.28E-06	0.005
biological process	in utero embryonic development	GO:0001701	346	2.04E-06	0.003
biological process	nervous system development	GO:0007399	1392	5.50E-08	< 1.0E-04

Table 2. Differentially expressed genes which clustered in "oxidation-reduction process" GO:0055114, "regulation of stress-activated MAPK cascade" GO:0032872, "*in utero* embryonic development" GO:0001701, and "nervous system development" GO:0007399 categories. p-values were calculated gene-wise using a two sample *t*-test and fold EtOH/Ctr represents the fraction between the mean expression value in the ethanol-exposed versus control embryos.

GO:0055114

Gene name	Gene description	p-value	Fold EtOH/Ctr
Dhcr24	24-dehydrocholesterol reductase	0.006	1.13
Fads2	fatty acid desaturase 2	0.018	1.13
Adh1	alcohol dehydrogenase 1 (class I)	0.026	1.12
Gpd1l	glycerol-3-phosphate dehydrogenase 1-like	0.037	1.15
Cp	ceruloplasmin	< 0.001	0.71
Blvrb	biliverdin reductase B (flavin reductase (NADPH))	< 0.001	0.80

Pnpo	pyridoxine 5'-phosphate oxidase	< 0.001	0.89
Snca	synuclein, alpha	0.004	0.85
Bdnf	brain derived neurotrophic factor	0.011	0.81
Ppp1r3c	protein phosphatase 1, regulatory (inhibitor) subunit 3C	0.017	0.87
Aifm2	apoptosis-inducing factor, mitochondrion-associated 2	0.028	0.87
Ppox	protoporphyrinogen oxidase	0.029	0.87

827 GO:0032872

Gene name	Gene description	p-value	Fold EtOH/Ctr
Mapk8ip1	mitogen-activated protein kinase 8 interacting protein 1	0.014	1.18
Nrk	Nik related kinase	0.021	1.18
Ephb1	Eph receptor B1	0.026	1.13
Mapk8ip2	mitogen-activated protein kinase 8 interacting protein 2	0.029	1.12
Ctgf	connective tissue growth factor	0.013	0.86
Ncor1	nuclear receptor co-repressor 1	0.016	0.77
Gadd45a	growth arrest and DNA-damage-inducible 45 alpha	0.030	0.89
Eda2r	ectodysplasin A2 receptor	0.044	0.85

828 GO:0001701

Gene name	Gene description	p-value	Fold EtOH/Ctr
Nrk	Nik related kinase	0.020	1.18
Epb4.1l5	erythrocyte protein band 4.1-like 5	0.020	1.11
Eif4e2	eukaryotic translation initiation factor 4E member 2	0.020	1.10
Wnt2	wingless-related MMTV integration site 2	0.042	1.11
Slit2	slit homolog 2	0.002	0.89
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	0.011	0.86
Maff	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	0.011	0.87
Adm	adrenomedullin	0.021	0.86
Cyr61	cysteine rich protein 61	0.028	0.89
Klf1	Kruppel-like factor 1 (erythroid)	0.028	0.79
Pdgfb	platelet derived growth factor, B polypeptide	0.037	0.89

829 GO:0007399

Gene name	Gene description	p-value	Fold EtOH/Ctr
Pitx1	paired-like homeodomain transcription factor 1	0.003	1.13
Clmn	calmin	0.005	1.14
Lrp8	low density lipoprotein receptor-related protein 8, apolipoprotein e receptor	0.008	1.16
Fzd3	frizzled homolog 3 (Drosophila)	0.011	1.14
Astn1	astrotactin 1	0.023	1.17
Ephb1	Eph receptor B1	0.026	1.13
En2	engrailed 2	0.027	1.28
Apcdd1	adenomatosis polyposis coli down-regulated 1	0.027	1.13

Ncdn	neurochondrin	0.027	1.11
Lpar3	lysophosphatidic acid receptor 3	0.027	1.11
Sall1	sal-like 1 (Drosophila)	0.028	1.12
Mapk8ip2	mitogen-activated protein kinase 8 interacting protein 2	0.029	1.12
Sphk2	sphingosine kinase 2	0.034	1.12
Pax7	paired box gene 7	0.038	1.11
Sox1	SRY-box containing gene 1	0.041	1.16
Pou3f1	POU domain, class 3, transcription factor 1	0.041	1.37
Wnt2	wingless-related MMTV integration site 2	0.042	1.11
Pcsk9	proprotein convertase subtilisin/kexin type 9	0.046	1.12
Prdm6	PR domain containing 6	0.047	1.13
Slit2	slit homolog 2	0.002	0.89
Tenm2	teneurin transmembrane protein 2	0.005	0.83
Limk1	LIM-domain containing, protein kinase	0.006	0.90
Arhgef28	Rho guanine nucleotide exchange factor (GEF) 28	0.008	0.86
Ntf3	neurotrophin 3	0.009	0.88
Cebpb	CCAAT/enhancer binding protein (C/EBP), beta	0.011	0.86
Gfra1	glial cell line derived neurotrophic factor family receptor alpha 1	0.011	0.79
Bdnf	brain derived neurotrophic factor	0.011	0.81
Nrxn1	neurexin I	0.013	0.86
Ncor1	nuclear receptor co-repressor 1	0.016	0.77
Ndrg1	N-myc downstream regulated gene 1	0.018	0.77
Trp63	transformation related protein 63	0.019	0.90
Adm	adrenomedullin	0.021	0.86
Dlc1	deleted in liver cancer 1	0.022	0.76
Ndrg2	N-myc downstream regulated gene 2	0.025	0.87
Itga1	integrin alpha 1	0.028	0.82
Foxd1	forkhead box D1	0.030	0.88
Etv1	ets variant gene 1	0.030	0.89
Sema3d	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	0.031	0.85
Ankrd1	ankyrin repeat domain 1 (cardiac muscle)	0.038	0.89
Lrrk2	leucine-rich repeat kinase 2	0.040	0.88

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832 **Highlights**:

833 - Fetal ethanol exposure increases neurons' vulnerability towards metabolic stressors

834 - Maternal alcoholism decreases offsprings' neuronal tolerance to hypoxia/ischemia

835 - Ethanol alters expression of genes associated with apoptosis and neuroprotection

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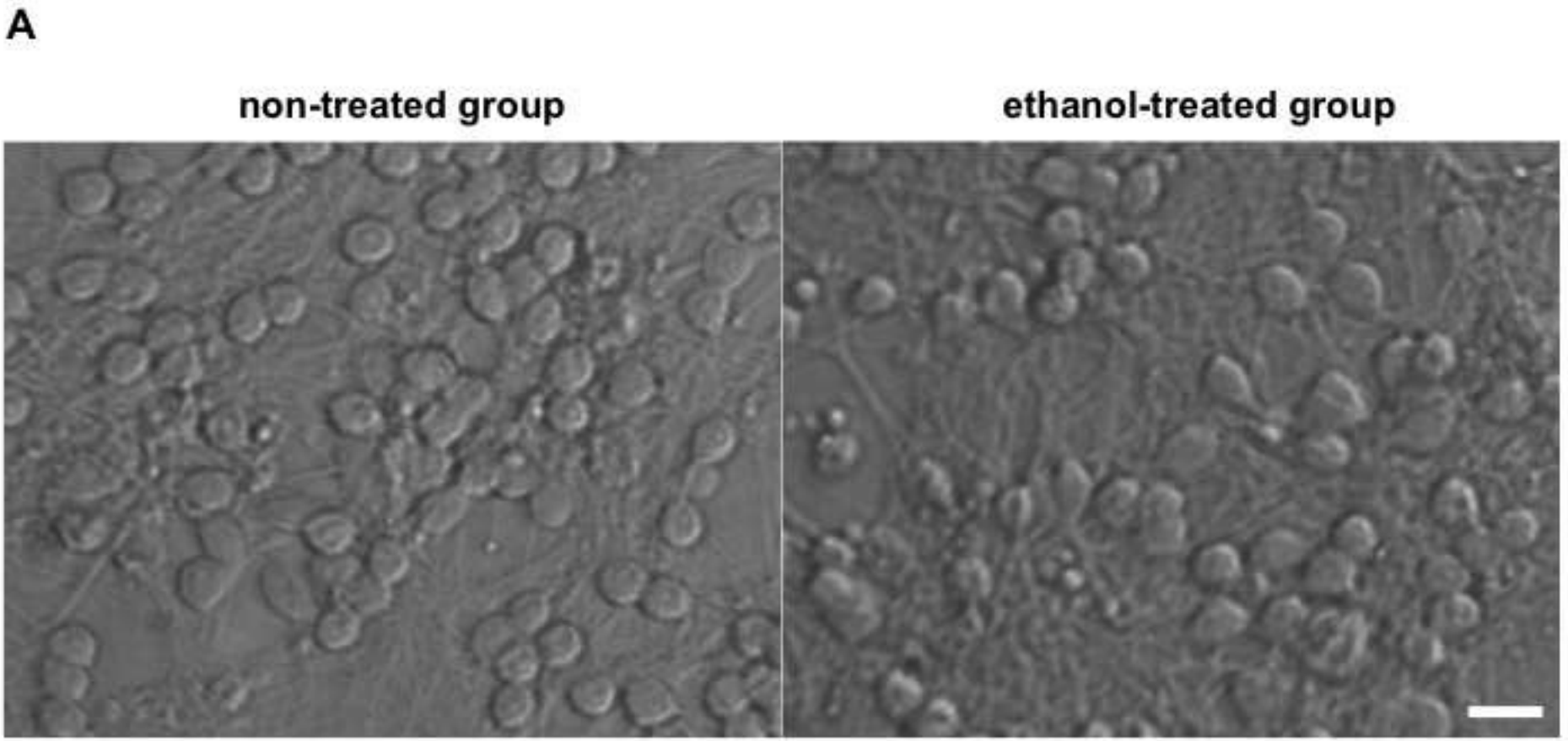
Figure



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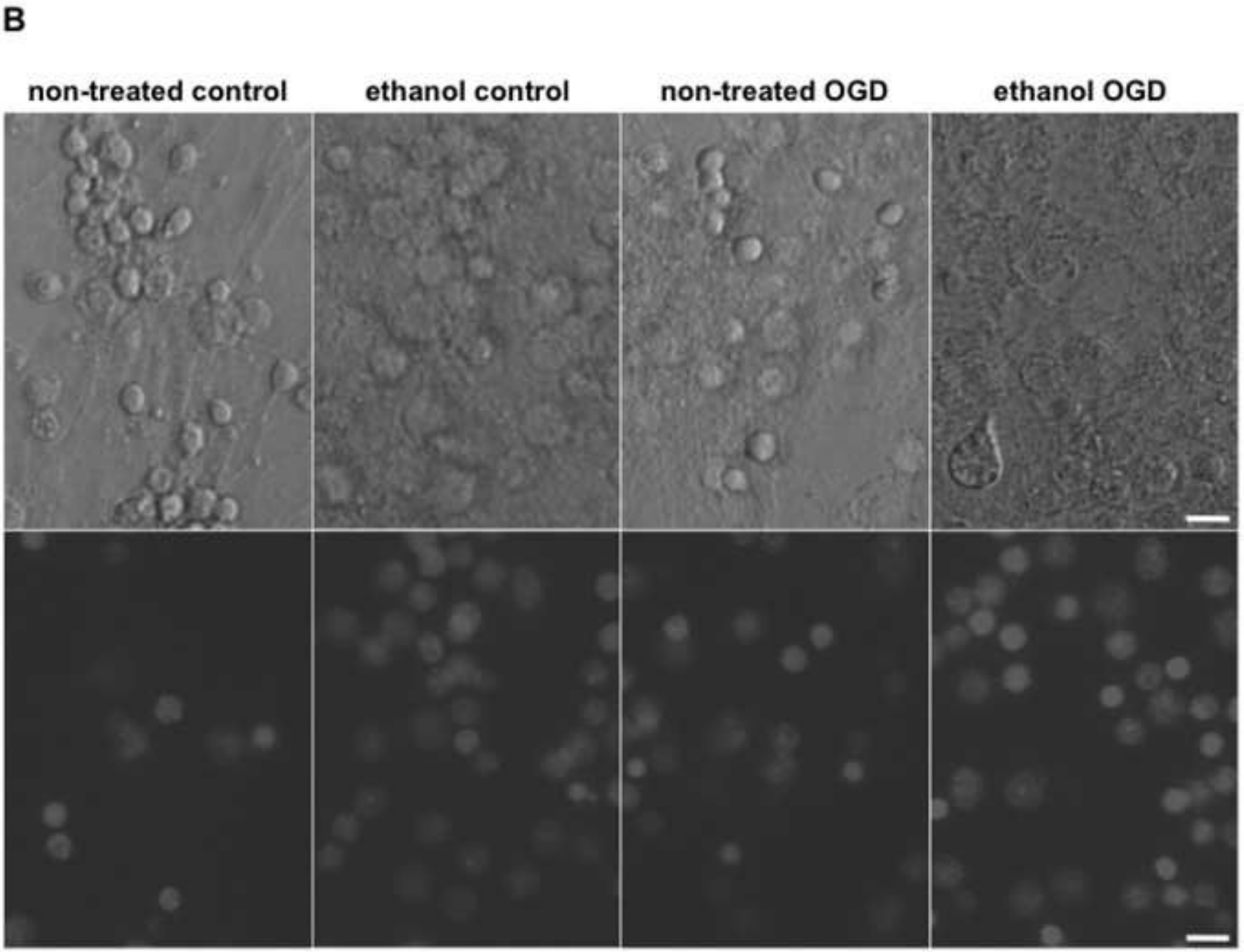

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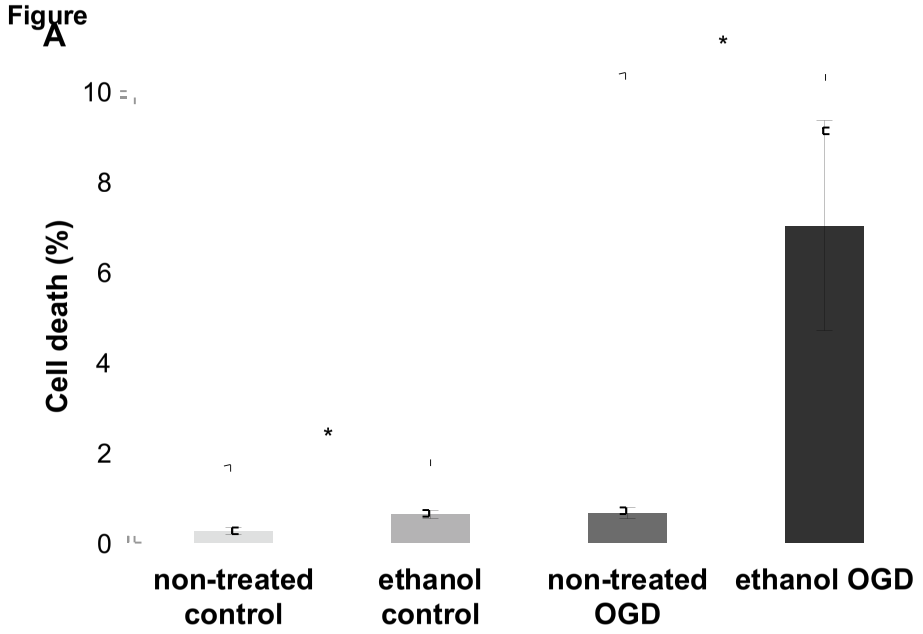
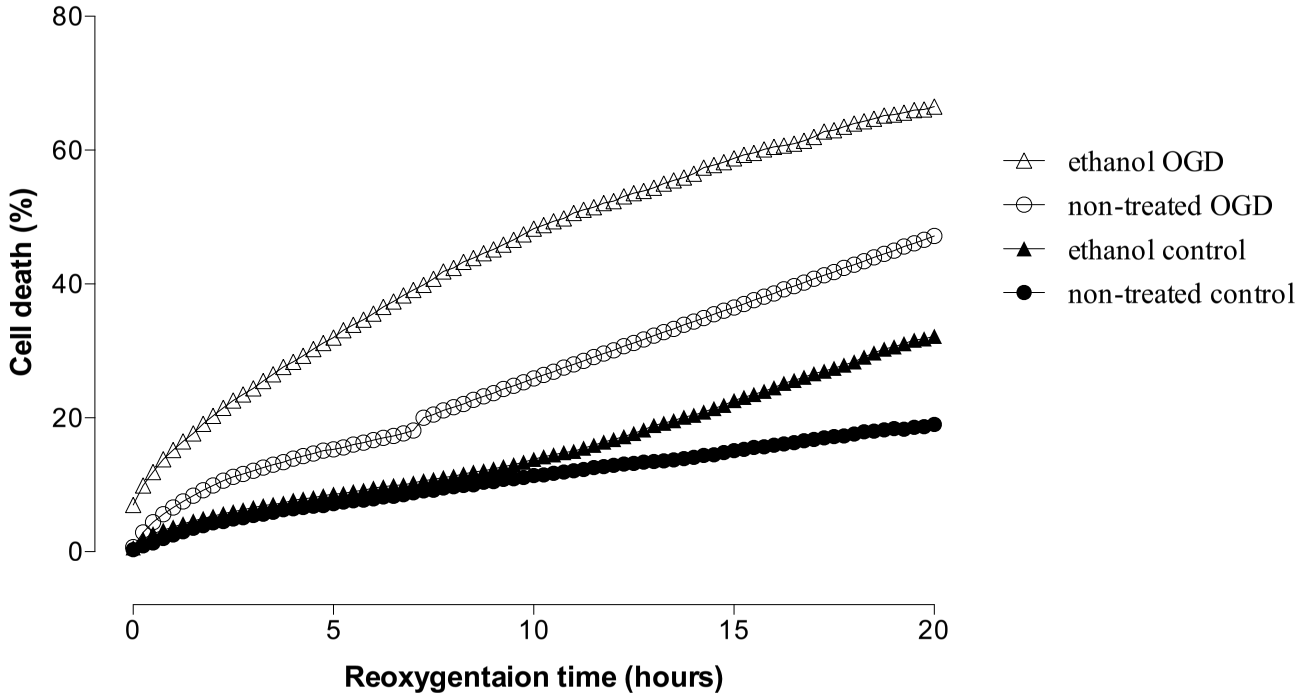


Figure 3

Figure

A



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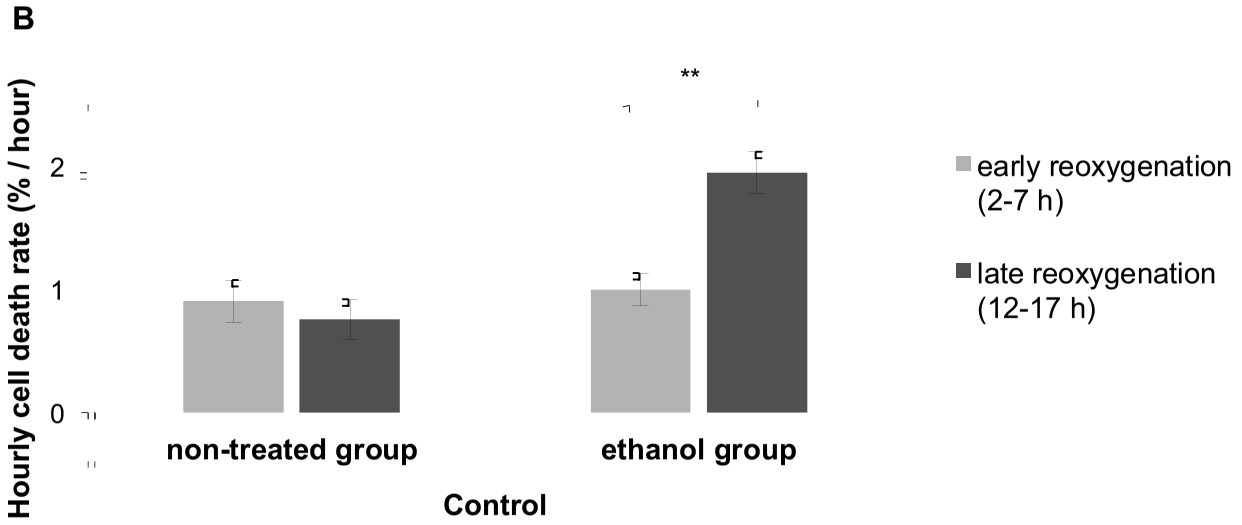

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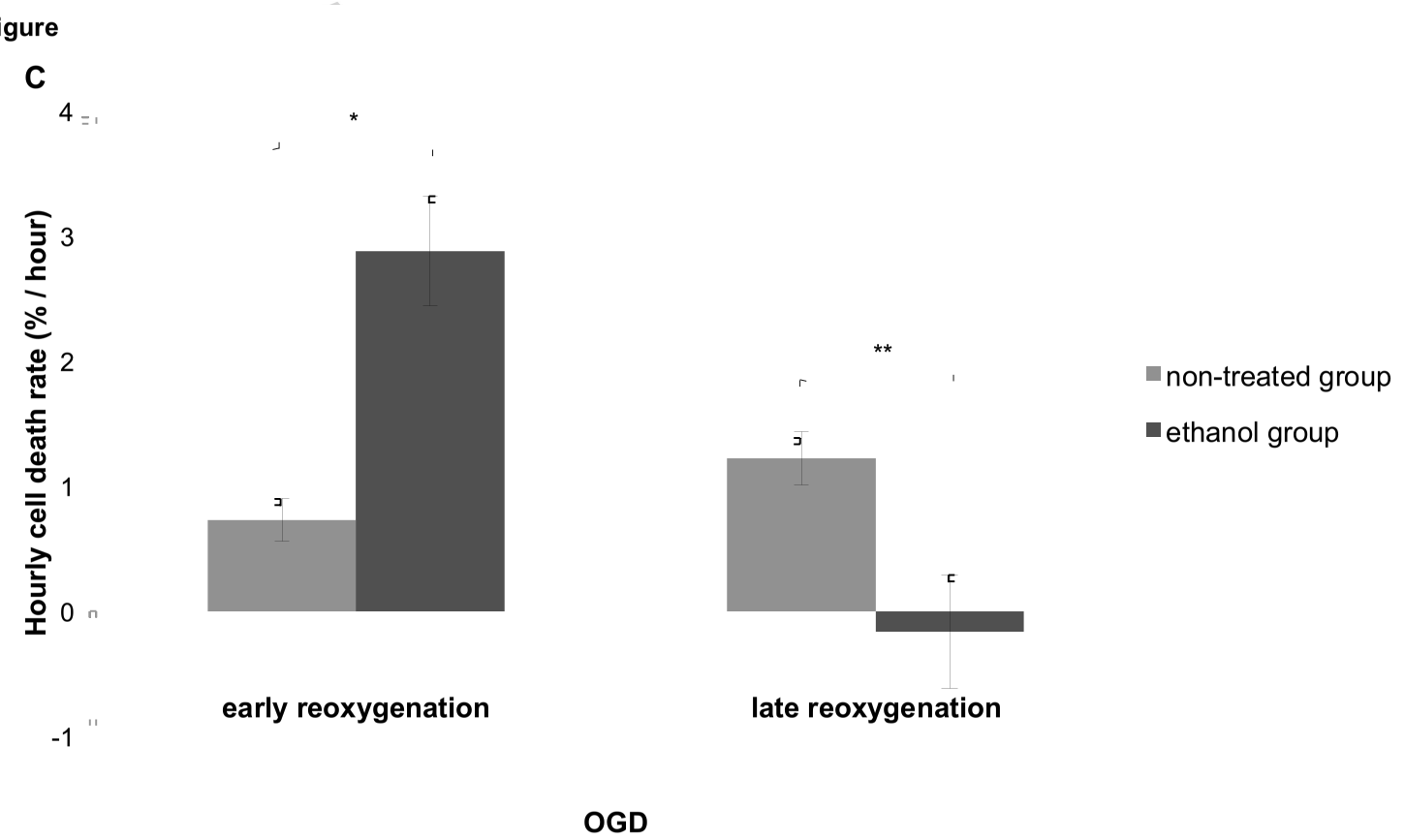

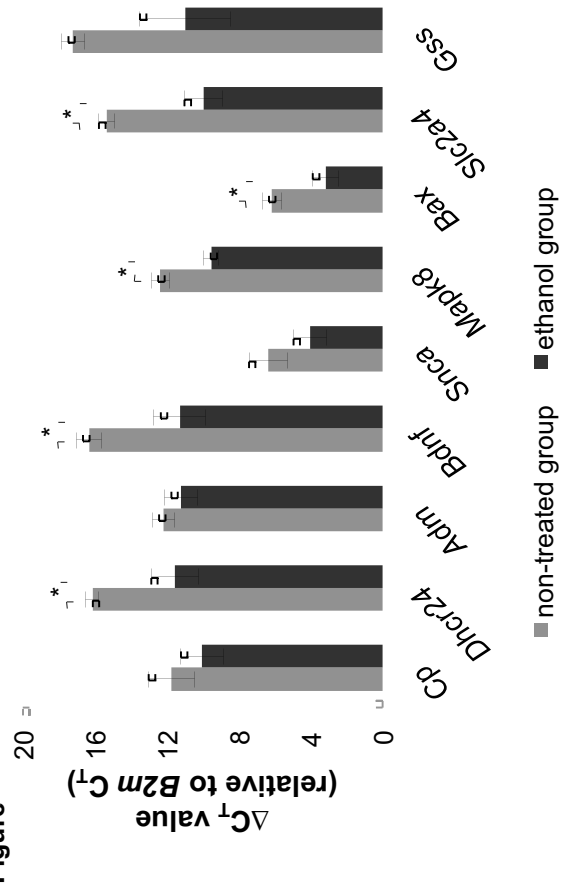

Figure